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(54) Title: METHOD FOR THE PRODUCTION OF TRANSGENIC CEREALS

(57) Abstract

The present invention relates to plant biotechnology and specifically to a new method for obtaining stable transgenic monocotyledonous plants, especially cereals, and to a method for obtaining transgenic cereal seeds. The present invention thus provides cereals which carry in their genome at least one foreign gene giving to the plant a desirable new trait, but which cereals do not carry any additional selectable genes in their genome. In the method of this invention a transgenic monocotyledonous plant is produced by providing a genetic construct comprising at least one gene heterologous to the plant to be transformed, whereby no additional selectable genes are inserted into the genetic construct, transforming the meristematic cells of the embryo of a monocot plant with the said genetic construct, and generating a whole plant by germinating and cultivating the transformed embryo without selection.

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METHOD FOR THE PRODUCTION OF TRANSGENIC CEREALS

Field of the Invention

The present invention relates to plant biotechnology and specifically to a new method for obtaining stable transgenic monocotyledonous plants, especially cereals, and to a method for obtaining transgenic cereal seeds. The present invention thus provides cereals which carry in their genome at least one foreign gene giving to the plant a desirable new trait, but which cereals do not carry any additional undesired selectable genes in their genome.

Background of the Invention

Recently there have been several reports of transformation of plant cells with genes of bacterial, plant or mammalian origin. It has been proposed to use whole plant tissues for production of heterologous protein material in plant hosts. Many of the transformation experiments have been made in tobacco plant, which can easily be transformed by *Agrobacterium* infection or electroporation of protoplasts (PCT application WO 91/02066). Tobacco plant can be regarded as a model system in plant transformation.

There is at present no satisfactory method available for routine transformation of barley or other cereals. Cereals belonging to the *Monocotyledonae* have proved to be very recalcitrant to genetic engineering. The *Agrobacterium*-mediated transformation routinely used for dicotyledonous plants has not been successful in transforming monocot plants. However, an application of *Agrobacterium*-mediated transformation of monocots is disclosed in WO 92/14828, wherein a method for genetic transformation of cereals is described, in which a bacterial vector carrying a foreign gene is cocultivated with a microspore culture of the plant to be transformed. The microspore culture is then regenerated in a selective medium.

The failure to achieve satisfactory *Agrobacterium*-induced transformation in cereals has led to increased interest in other methods for the production of transgenic plants, such as the use of DNA-coated microprojectiles and direct uptake of DNA into cells or protoplasts stimulated either electrically or chemically. In addition to the problem of delivering foreign DNA into the target cell there is the problem of the competence of the cell to integrate the DNA into its genome, regenerate to a new plant and produce progeny with the transferred new trait. This means that one basic requirement for the use of any of these methods is an efficient culture and regeneration system that guarantees a large scale regeneration of fertile plants from single transformed cells.

At the moment plant regeneration remains the major limitation to the application of genetic engineering of plant cells to crop improvement. The currently used gene transfer techniques require the use of embryogenic cultures, which are rather difficult to establish and maintain. The ability to produce such regenerable cultures is not only a species specific but also a cultivar specific feature.

A new application of electroporation may overcome at least some of these problems. Dekeyser *et al.* (1990) have demonstrated transient expression of genes delivered into leaf segments of different cereal species by electroporation. In PCT-application WO 92/09696 the same method is applied to obtain transgenic maize plants from immature zygotic embryos or type I embryogenic callus. To prepare them to take up DNA, the callus or immature embryos are first wounded, either mechanically or chemically. Foreign DNA is then transferred to them by electroporation. The electroporated embryos or callus are then cultured for several weeks on selective medium before resistant calli are grown into plants. The method is based on the ability to produce embryogenic callus from the scutellar tissue of immature embryos.

Particle bombardment is another gene transfer method which has been successfully used in transforming cereals. In the PCT application WO 90/01551 transformation of monocot plants with exogenous DNA to produce foreign proteins is described.

The PCT-application WO 91/02071 discloses a system for genetic transformation of monocotyledonous plant cell suspension, e.g. maize cell suspension, by particle bombardment and the selection of genetic transformants from the suspensions. Fertile plants are then produced from the transformed cells by conventional methods. Particle bombardment has also been used to produce several transgenic rice varieties by transforming the scutellar tissue of immature embryos (Christou *et al.* 1991). In addition to the "traditional" biolistic apparatus there is a new micro-targeting device for particle bombardment of plant meristems (Sautter 1993), but so far tobacco is the only transgenic plant produced with this method.

Eventhough there has been considerable success lately in the field of cereal transformation, none of the published methods offer a general routinely applicable method for obtaining large numbers of transformed plants. In comparison to the earlier ones the present invention offers a relatively fast and simple method for producing transgenic cereal plants, which method furthermore is universally applicable to all cereals independent of genotype or cultivar.

In a grass embryo there are several separately transformable meristematic regions. A grass embryo also reaches a relatively high degree of differentiation (e.g. Esau, 1977). For example in large barley seeds there are nine meristems leading to nine mutually exclusive mutant sectors, and up to seven additional meristems leading to mutually exclusive mutant sectors can be present in the embryo of the seed (Jacobsen, 1966, see Fig. 1). In an embryo independent mutant sectors which cannot have a mutant cluster in common, are proven anatomically, if a shoot or shoot group exists as a separate shoot primordium or as a meristematic tissue area separated from the apex of the shoot primordium by a leaf or leaf primordium. The anatomical data from barley embryos proves that the embryo contains at least six separate shoot meristems or prospective shoot meristems which will constitute mutually exclusive mutant sectors in the plant (Jacobsen 1966). Similar results have been reported for rice in which the spikes on the primary side-shoots mutate independently of the apical spike. The present invention is developed to utilize the whole potential of the numerous meristems of the grass embryo. The present in-

vention utilizes conventional gene transfer methods, the gist of the system lying in the treatment of the plant material.

Summary of the Invention

The present inventors have now developed a new efficient method for transforming grasses and cereal plants. The method of the invention is based on the fact that a grass embryo has several separately transformable meristematic regions. E.g. a barley grain has, depending on its size and stage of development, even 16 separately transformable meristems. The transformation is thus directed to such a region of the embryo in which the leaf primordia and the side shoot primordia or their initial cells are located.

The transformed embryos are germinated and cultivated directly without any selection, and the rachises and awns of the ears produced are analyzed as regards the transferred genes. A special advantage of the present method is thus the fact that no selection is used when growing the transformed embryo material into plants so as to exploit the whole growing capacity of the meristematic region. Consequently, it is not necessary to insert selectable genes into the genetic construct used for the transformation of the embryonic material. Another special advantage of the method of the invention is the minimal requirement of tissue culture, and it helps to eliminate culture induced mutations and somaclonal variation. Thus the transformed embryos can be germinated directly without the laborious cell cultivation step. The direct germination of the transformed embryos also makes it possible to use varieties that are difficult to regenerate in tissue culture.

It is therefore an object of this invention to provide a method for producing a transgenic monocotyledonous plant by providing a genetic construct comprising at least one gene heterologous to the plant to be transformed, whereby no additional selectable genes are inserted into the genetic construct, transforming the meristematic cells of the embryo of a monocot plant with the said genetic construct with

direct gene transfer methods, and generating a whole plant by germinating and cultivating the transformed embryo without selection.

A further object of the invention is to provide a method for generating transgenic cereal seed comprising generating transgenic plants by the above method, cultivating green plantlets and deriving seed from the resulting mature plants.

The plants as well as seeds obtained by the above defined methods are also objects of the present invention.

Detailed Description of the Invention

It has now been found that certain modifications in relation to the selection of transformable material and the culture technique enable significantly enhanced efficiency to be achieved in grass and cereal transformation. The method of the invention to produce a transgenic monocot plant is applicable to grasses and cereals, primarily barley, *Hordeum vulgare* L., but can be used also for e.g. wheat, oat, rye, rice or maize. Accordingly, the present invention provides a method for generating transformed cereal plants comprising transforming the embryo material and growing it directly into plants, wherein the growth medium does not comprise any selective substances for the transformed trait.

The material to be transformed must be an embryo, either zygotic or somatic, immature or mature and it preferably contains several separate meristematic regions to be transformed. Parts of an embryo, particularly the nodal region, or intact embryos can be transformed. The term embryo or embryo material should be understood to include all stages of development from the globular stage to the mature stage of zygotic and somatic embryos. The embryos may be transformed directly after isolation or after being germinated for 1 to 5 days prior to the transformation.

To demonstrate the method of the invention the β–glucuronidase enzyme (GUS) and the neomycin phosphotransferase enzyme (NPTII) were used as an example because there are suitable assays to detect their activity in plant tissues. The cDNA coding for β–glucuronidase under control of the maize alcohol dehydrogenase I promoter was transferred by particle bombardment to the meristematic cells of immature barley embryos and mature embryos of barley, wheat and maize to demonstrate the general applicability of the method to different cereal species. A histological assay for β–glucuronidase was performed for the transformed embryos. The blue stain indicating the transformation events was localized in the meristematic regions of the embryos by serial sectioning of the samples. The cDNA coding for neomycin phosphotransferase was placed under control of the cauliflower mosaic virus 35S promoter and transferred by particle bombardment to the meristematic cells of barley embryo. The transformed embryos were germinated and cultivated without selection so as to produce as many shoots as possible. The rest of the spike after grain removal were then analyzed as regards the transferred genes, and grains of the positive ears were used for the production of several generations of transgenic barley plants.

To demonstrate the use of the method for the production of transgenic cereal plants using other gene constructions immature embryos of barley were transformed with the plasmid pKAH24 containing the *egl1* gene coding for endo–β–glucanase I of *Trichoderma reesei* under control of the barley α–amylase promoter. The transformed embryos were germinated and cultivated without selection to produce as many shoots as possible. 9 of the 146 plants analyzed by the polymerase chain reaction contained the transferred gene.

The method of this invention can also be used to produce transgenic cereal plants expressing any other desirable new trait. Especially interesting for barley are enzymes of importance in brewing such as β–glucanases and amylolytic and proteolytic enzymes. For barley and wheat especially interesting are some agronomically important characteristics like preharvest sprouting. Cereal grains differ in their nutritional characteristics. The value of cereal protein is less than that from

animal products mainly because of deficiencies of several essential amino acids. The food and feed value of wheat and barley grains can be improved with genetic engineering of their storage protein genes. The method of this invention can also be used to produce transgenic plants that produce other foreign products, such as pharmaceutical peptides, proteins or compounds of secondary metabolism.

Direct gene transfer methods are used for the transformation. Suitable methods are e.g. particle bombardment and electroporation, electrophoresis may also be used. The transformed embryos or embryo parts are germinated on modified MS medium without selection so as to allow all the potentially transformed meristems to form shoots. At this stage the growth of the tillers can be enhanced, if desired. The enhancement can be effected e.g. by cutting the main shoots of the plants after sprouting. Later the rooted plantlets are grown in soil for seed production. The rachises of the ears produced are then analyzed as regards the transferred genes, and grains of the positive ears are used for the production of transgenic cereals.

The seeds from the cultivated green plantlets may be derived by harvesting them directly from the resulting mature plants or by obtaining seeds from plants one or more generations remote from the original green plantlets.

Abbreviations

T_0 primary transformant

T_1 first progeny of T_0

Brief Description of the Drawings

Fig. 1 Diagram of a transversely cut barley embryo.

Fig. 2A The vector pKAH24 containing the endo–β–glucanase gene *egl1*.

Fig. 2B The vector pKAH21 containing the endo–β–glucanase gene *egl1*.

Fig. 2C The vector pHTT303 containing the neomycin phosphotransferase gene *nptII*.

Fig. 2D The vector pAT13 containing the β–glucuronidase gene *uidA*.

Fig. 3 (A–C) Histochemical localization of β–glucuronidase gene in transformed immature embryos of barley.

Fig. 4 (A–D) Histochemical localization of β–glucuronidase gene in transformed mature embryos of barley.

Fig. 5 (A–D) Histochemical localization of β–glucuronidase gene in transformed mature embryos of wheat.

Fig. 6 (A–C) Histochemical localization of β–glucuronidase gene in transformed mature embryos of maize.

Fig. 7 Screening for *egl1* by PCR technique.

Fig. 8 NPTII gel assay of leaf extracts from T_0 and T_1 barley plants. Lane PC represents a positive control from tobacco transgenic for 35S–*nptII* and lane NC a negative control from a non–transformed barley plant. Samples I to IV represent four spikes of the T_0 plant *Toivo* and samples 1 to 25 their progeny (T_1), so that 1 to 7 are the offspring of I, 8 to 14 are offspring of II, 15 to 18 offspring of III, and 19 to 25 offspring of IV. Samples are numbered so that they are equivalent in Figures 8, 9 and 10.

Fig. 9 Southern blot analysis of transgenic T_0 and T_1 plants. *Bam*HI, *Eco*RI and *Hind*III digested total DNA samples (5 μg) were probed with plasmid pHTT303. The positive control (PC) is *Bam*HI, *Eco*RI and *Hind*III digested pHTT303 (5 pg, representing approximately one copy per haploid genome), mixed with 5 μg

*Bam*HI, *Eco*RI and *Hind*III digested DNA from a non-transformed barley. This digestion releases vector (2.6 kb), *nptII* gene (1.7 kb) and 35S promoter (1.0 kb) fragments. The S and L lanes represent negative controls containing DNA isolated from non-transformed barley spikes and leaves, respectively. Samples II to IV represent spikes of the T_0 plant *Toivo* and samples 8 to 25 their progeny (T_1). Samples are numbered so that they are equivalent in Figures 8, 9 and 10. The intensity of the bands in the barley samples as well as the number of integration specific fragments indicate that about two copies of pHTT303 were integrated into the barley genome. Note that part of the T_0 spikes may be chimeric and part of the T_1 plants homozygous for the transferred DNA, resulting in some variation of band intensities between the samples.

Fig. 10 Analysis of methylation pattern of the transferred DNA in T_1 plants. *Bam*HI, *Eco*RI and *Hind*III digested total DNA samples (5 µg) were in addition restricted with either *Dpn*I (D1) or *Dpn*II (D2) and probed with plasmid pHTT303. The positive control (PC) is *Bam*HI, *Eco*RI and *Hind*III digested pHTT303 that was further restricted with either *Dpn*I (D1) or *Dpn*II (D2). 5 pg of digested plasmid was mixed with 5 µg DNA from non-transformed barley, digested with same enzymes as the plasmid. The S lane represents the negative control containing DNA isolated from non-transformed barley plant spikes. The three T_1 plant samples 9, 15 and 22 are offspring of T_0 spikes II, III and IV, respectively. See text for conclusions.

Experimental

Unless otherwise stated all experiments described herein are effected according to conventional procedures used in genetic engineering.

Example 1. Construction of chimaeric genes for expression in plants.

pKAH24 and pKAH21 (expressing endo–β–glucanase)

The 5.2 kb plasmid consists of the barley α–amylase promoter and the gene *egl1* of *Trichoderma reesei* coding for the endo–β–glucanase I in the *E. coli* cloning vector pUC18 (Norrander *et al.*, 1983). The plasmid pKAH21 contains the same *egl1* gene under control of cauliflower mosaic virus 35S promoter. The plasmid constructs pKAH24 and pKAH21 are shown in Figures 2A and 2B.

pHTT303 (expressing neomycin phosphotransferase II)

The gene *nptII* coding for neomycin phosphotransferase II was combined at its 5' end to the cauliflower mosaic virus 35S promoter and at its 3' end to a terminator of the gene 7 of T–DNA. The recombinant gene resides in the *E. coli* cloning vector pUC18 (Norrander *et al.*, 1983) as a *Bgl*II–*Sac*I fragment between the *Bam*HI and *Sac*I sites of the vector. The plasmid construct pHTT303 is shown in Figure 2C.

pAT13 (expressing β–glucuronidase)

The plasmid pAT13 was a gift from Carlsberg Research Institute. The 6.5 kb plasmid consists of the alcohol dehydrogenase 1 promoter, a fragment from intron 1 of the *Adh* I gene, a β–glucuronidase coding region, and the nopaline synthase polyadenylation region inserted into pUC8 (Klein *et al.* 1988), as pAI_1 GusN. The plasmid construct pAT13 is shown in Figure 2D.

Example 2. Transformation of immature embryos of barley by particle bombardment.

Immature embryos of the size of 0.5 to 1 mm were isolated from developing seeds of barley (*Hordeum vulgare* L. cv. Kymppi). The embryos were bombarded at the embryonic axis. For particle bombardment the isolated embryos were placed scutellum down on modified MS medium (Olsen, 1987) supplemented with 0.4 mg/l BA and 35 g/l maltose and solidified with 3 g/l gellan gum. The principle of particle bombardment is described by Sanford *et al.* (1987) and Klein *et al.* (1987). The helium modification of the Biolistic® PDS–1000 device (Bio–Rad) was used for transformation. The following conditions were used: sample chamber pressure 3.1 kPa; pressure of helium shock wave 9.0 MPa; distance between macrocarrier and stopping screen 6 mm; distance between macrocarrier and rupture disk 9 mm. Particles were prepared by coating gold particles (1.0 µm in diameter) with the plasmid pKAH24 (β–glucanase), or with the plasmid pAT13 (β–glucuronidase), or with a 1:1 mixture of plasmids pHTT303 (neomycine phosphotransferase) and pAT13. In each bombardment, 1.0–2.0 µg of DNA was used and each sample was bombarded once.

Example 3. Transformation of mature embryos of barley, wheat and maize by particle bombardment.

Mature seeds of barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) were surface sterilized first with 70% ethanol for 5 min and then with NaOCl (4% active Cl) for 10 min, rinsed several times with sterile distilled water and germinated on moist filter paper for 1 day. The embryos were separated from the seeds and part of the coleoptile and leaves covering the meristematic regions of the embryo were removed with a scalpel. The embryos were transformed by particle bombardment with the plasmid pAT13 as described in Example 1.

Example 4. Histochemical localization of the β-glucuronidase gene.

Immature embryos of barley and mature embryos of barley, wheat and maize transformed with the plasmid pAT13 were stained with X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt) with the method of Jefferson (1987) with the modifications of Ritala *et al.* (1993) to detect the activity of the transferred *uidA* gene coding for β-glucuronidase. The stained embryos were embedded in paraffine and sectioned with microtome to localize the transformed cells. The localization of blue stain indicating transformation in the meristematic regions is shown in Figure 3 (3A-3C) for immature embryos of barley, Figure 4 (4A-4D) for mature embryos of barley, Figure 5 (5A-5D) for mature embryos of wheat, and Figure 6 (6A-6C) for mature embryos of maize.

Example 5. Growing the transformed plants.

After transformation the embryos were transferred on modified MS medium (Olsen 1987) supplemented with 0.4 mg/l BA and 35 g/l maltose and solidified with 3 g/l gellan gum for germination. The plates were incubated at 23°C in light (50 µmol m^{-2} s^{-1}). The embryos were germinated without any selective substances in the medium. After three weeks the shoots were transferred to modified MS rooting medium (Olsen 1987) without hormones and solidified with 3 g/l gellan gum. After 2 to 3 weeks the plantlets were potted in soil and grown to maturity in the greenhouse. The embryos from all green grains were isolated for further cultivation, the rachises and/or awns were screened for the presence of the transferred gene.

Example 6. Screening for the transformed plants.

Assay for NPTII activity. Neomycin phosphotransferase II activity in the plants was assayed according to McDonnell *et al.* (1987) with the following modifications: Extraction buffer with 0.13 g/l leupeptine and a small amount of purified sea sand was added to the leaf material which was then ground thoroughly in an Eppendorf tube and centrifuged. The supernatant was collected and the protein con-

centration determined (Bradford, 1976). The amount of extract used in the enzyme assay corresponded to 20 µg of total protein. The reaction mixture was blotted after 5 minutes centrifugation via a dot blot apparatus (Milliblot–D, Millipore) onto Whatman P81 paper. Alternatively, the NPTII gel assay (Reiss *et al.* 1984, modified by Van den Broeck *et al.* 1985) was used.

Screening of plants by PCR technique.

Screening for *egl1*. The plant material was screened by the polymerase chain reaction (PCR) carried out in a Perkin Elmer cetus 9600 thermocycler. The complete PCR mixture contained 100–500 ng of genomic or 0.5 pg of pKAH21 DNA, 12.5 pmol of each oligonucleotide primer, 200 µM dNTPs, 0.5 U Dynazyme and buffer supplied by the enzyme manufacturer (Finnzymes Oy) in a total volume of 100 µl. Thirty cycles were performed under following conditions: 75 s at 95°C, 2 min at 55°C, and 3 min at 72°C. Primers were designed to amplify a 557 bp fragment of the cDNA for EGI. The forward primer was 5'–AGGACACCTCGGTGG–TCCTT–3' and the reverse primer 5'–AGAGTGAGGGGTCAAGGCATT–3'. The PCR performance was controlled by including a primer pair amplifying the promoter fragment of one of the α–amylase genes. Total DNA isolated from a transgenic barley cell line (VTT–6–93002, transformed with pHTT303 and pKAH21) was used as a positive control. The amplified samples were analyzed by electrophoresis in a 2% agarose gel.

Plants grown from immature embryos transformed with the plasmid pKAH24 were screened for the gene *egl1* by PCR. About 1400 spikes were analyzed from 146 plants. Nine plants of these (12 spikes) were shown to contain the transferred gene (Fig. 7).

Screening for *nptII*. The plant material was screened by the polymerase chain reaction (PCR) carried out in a Techne PHC–2 thermocycler. The complete PCR mixture contained 100–500 ng total or 0.15 pg pHTT303 DNA, 50 pmol of each oligonucleotide primer, 200 µM dNTPs, 2 U Dynazyme™ and buffer supplied by the enzyme manufacturer (Finnzymes Oy) in a total volume of 100 µl. Thirty

cycles were performed under following conditions: 75 s at 94°C, 2 min at 55°C, and 3 min at 72°C. Primers were designed to amplify a 5' sequence of *nptII* in pHTT303. The forward primer used was 5'-ACACGCTGAAATCACCAGTCTC-3' (+1 from start of transcription) and the reverse primer 5'-CTCGTCCTGCAGTTC-ATTC-3' or 5'-TCGCCCAATAGCAGCCAGTC-3' (+281 and +417 from start of transcription, respectively). The PCR performance was controlled by including a primer pair amplifying the promoter fragment of one of the α-amylase genes. The amplified samples were analyzed by electrophoresis in a 2% agarose gel.

During *in vitro* rooting, NPTII activity was analyzed from the 227 plants of the T_0 generation by the dot blot method. One plantlet proved to be NPTII positive, and this plant was named and is later referred to as *Toivo*. *Toivo* was transferred to potting soil and grown in the growth room. By now it has produced 98 fertile spikes. Embryos from green grains were isolated for further cultivation to T_1 progeny. The rest of the spikes of *Toivo* after grain removal were screened for the presence of the *nptII* gene with the PCR technique. Of the 90 spikes screened, six were positive for the *nptII* gene. Four of them gave positive signals in the NPTII gel assay (Fig. 8) as well. The presence of the transferred *nptII* gene in these four was confirmed with Southern blot hybridization (Fig. 9).

Screening of the T_1 generation

Progeny of four shoots of *Toivo* carrying the *nptII* gene were studied further. They produced twenty five plantlets as T_1 progeny. PCR analysis indicated that among the sets of siblings five out of seven, two out of seven, four out of four and four out of seven, contained the *nptII* gene. The NPTII enzyme activity analysis revealed that fourteen of them also expressed the transferred gene (Fig. 8). The integration of the *nptII* gene in the genomes of T_1 progenies was confirmed by Southern blot hybridization (Fig. 9).

Example 7. Southern blot analysis of T_0 and T_1 plants as evidence for transformation and inheritance.

Total DNA from the plant material was isolated according to Dellaporta *et al.* 1983. For Southern blots, isolated total DNA was restricted with enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Dpn*I and/or *Dpn*II in the buffers recommended by the enzyme manufacturers. 5 µg digested DNA was separated by electrophoresis in 0.8 % agarose gels. The gels were blotted onto MagnaGraph (Micron Separation Inc.) nylon membranes (Southern 1975). The ^{32}P–dCTP –labeled plasmid pHTT303 was used as a probe. Hybridizations (at 42°C overnight in 50 % formamide) and washing of the filters were carried out according to Sambrook *et al.* (1989). After washing the filter was exposed to X–ray film using intensifying screens at –70°C for three days.

Total DNAs isolated from the rest of the spikes of *Toivo* (T_0 generation) after grain removal and from leaves of the T_1 progeny were analyzed for the presence of the *nptII* gene. The isolated DNAs were digested simultaneously with the enzymes *Bam*HI, *Eco*RI and *Hind*III. This releases vector (2.6 kb), *nptII* (1.7 kb) and 35S promoter (1.0 kb) fragments. When hybridized with the pHTT303 probe, the *Bam*HI–*Eco*RI–*Hind*III –digested total DNA sample of the non–transformed control shows a 2.6 kb band as background. In addition to this unspecific band (that overlaps with the vector band), the DNA samples from the four T_0 shoots and from fifteen T_1 progeny shoots contained copies of the *nptII* and promoter fragments, and in addition several extra fragments (Fig. 9). The extra fragments do not correspond to partial digestion fragments of pHTT303. This indicates integration of the transferred pHTT303 into the genome of *Toivo* as well as passage of both the transferred sequences and the integration pattern to T_1 progeny. From this fact it is also apparent that all of the transgenic tillers originate from a single transformed cell and show integration at one locus.

In order to further confirm that the DNA hybridizing to pHTT303 is not carry-over of the plasmid DNA used for transformation, we performed analysis with the restriction enzymes *Dpn*I and *Dpn*II. They both recognize and cleave the sequence GATC, but while *Dpn*II cleavage is inhibited by the methylation of the A residue, *Dpn*I requires methylation. Our *E. coli* host DH5α performs methylation of the A residue of the GATC sequence, as do all strains wild type for the *dam* locus. However, the A residue is not methylated in eukaryotes (Torres et al. 1993). Figure 10 shows that the pHTT303 plasmid isolated from *E. coli* is cleaved by *Dpn*I but not *Dpn*II. On the other hand, the sequences homologous to pHTT303 in the barley DNA are cleaved by *Dpn*II but not *Dpn*I, indicating that their methylation status is different from that of the plasmid. We conclude that the pHTT303 homologous sequences in the barley samples have been replicated by the barley cells.

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Claims

1. A method for generating a transgenic monocotyledonous plant, comprising transforming the embryo material of the host plant and growing it directly into plant, wherein the growth medium does not comprise any selective substances for the transformed trait.

2. A method for producing a transgenic monocotyledonous plant, comprising

a) providing a genetic construct comprising at least one gene heterologous to the plant to be transformed, whereby no selectable genes are inserted into the said genetic construct,

b) transforming meristematic cells of the embryo of the host plant with the said genetic construct, and

c) generating a whole plant by germinating and cultivating the transformed embryo without selection.

3. A method according to claim 1 or 2 wherein the monocotyledonous plant is barley.

4. A transgenic monocotyledonous plant or a progeny thereof comprising at least one gene heterologous thereto stably integrated into its genome and being obtainable by the process of claim 1 or 2.

5. The transgenic monocotyledonous plant or a progeny thereof according to claim 4 wherein the plant is barley.

6. A method for generating transgenic cereal seed comprising generating transgenic plants by the method according to claim 1 or 2, cultivating green plantlets and deriving seed from the resulting mature plants.

7. A seed of a transgenic monocotyledonous plant, said seed comprising at least one gene heterologous to the said plant stably integrated into its genome and being obtainable by the method of claim 6.

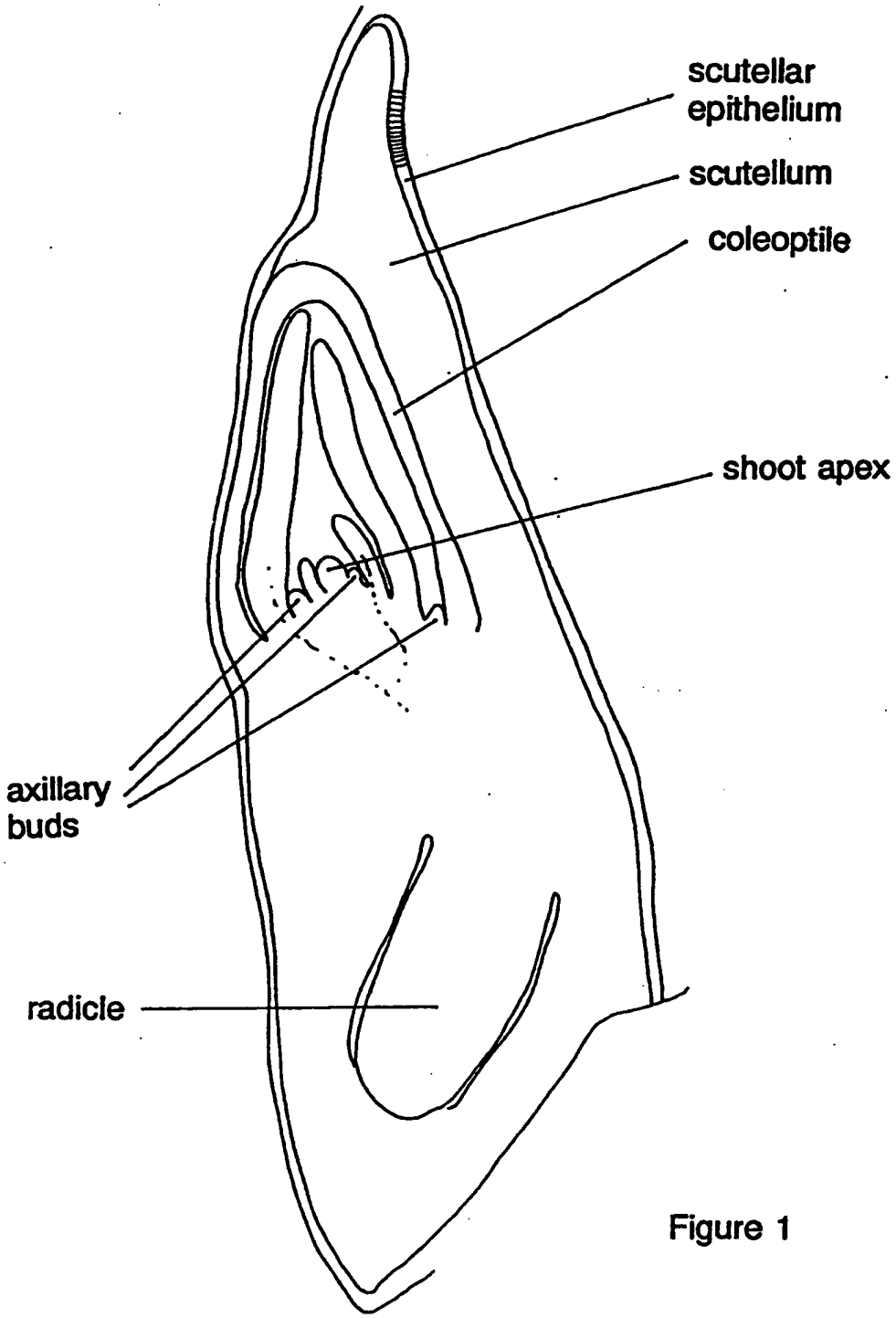
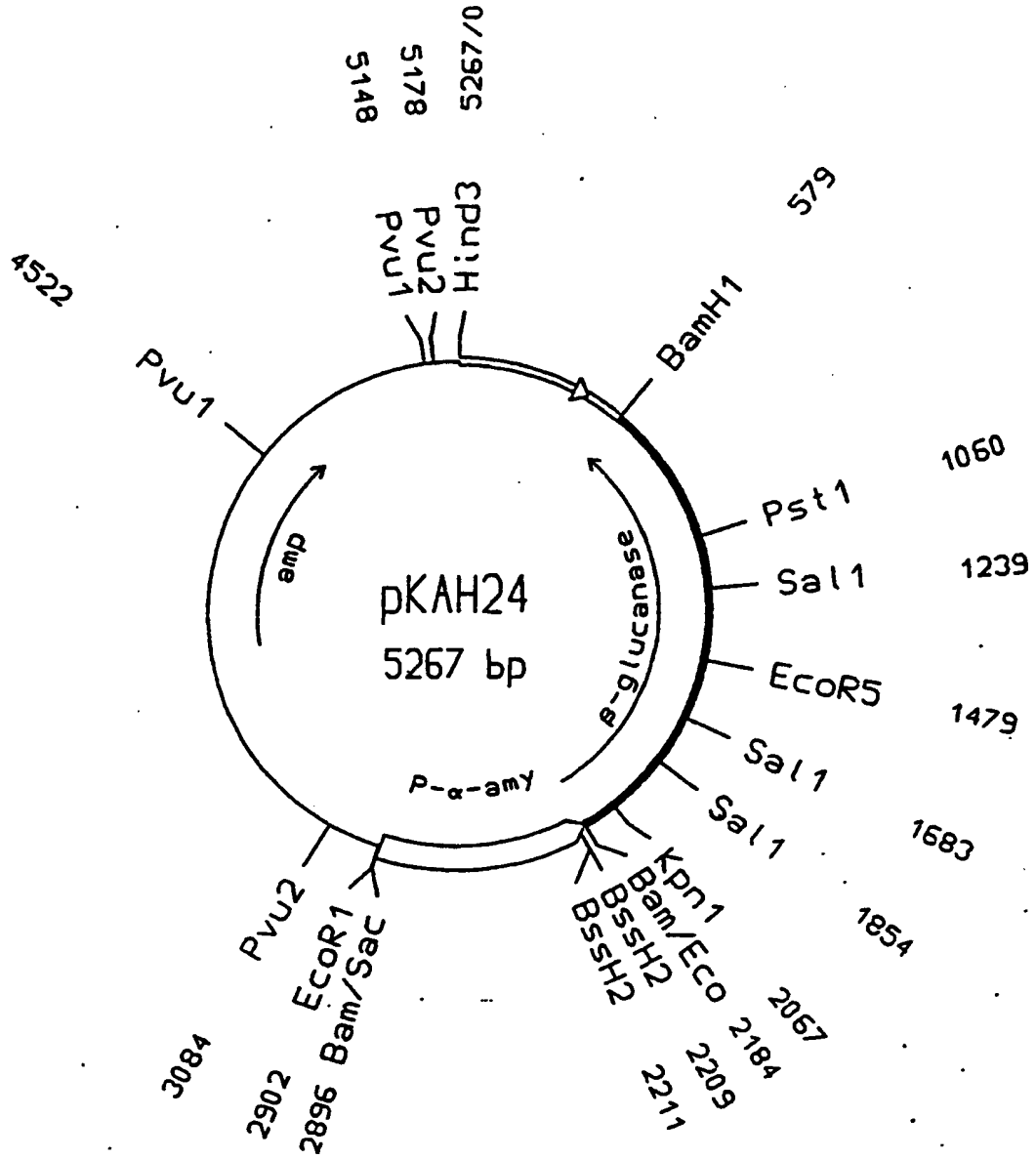

scutellar epithelium
scutellum
coleoptile
shoot apex
axillary buds
radicle

Figure 1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Pvu1
BamH1
579
1060
Pst1
Sal1
1239
EcoR5
1479
Sal1
Sal1
1683
Kpn1
Bam/Eco
BssH2
BssH2
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Pvu1
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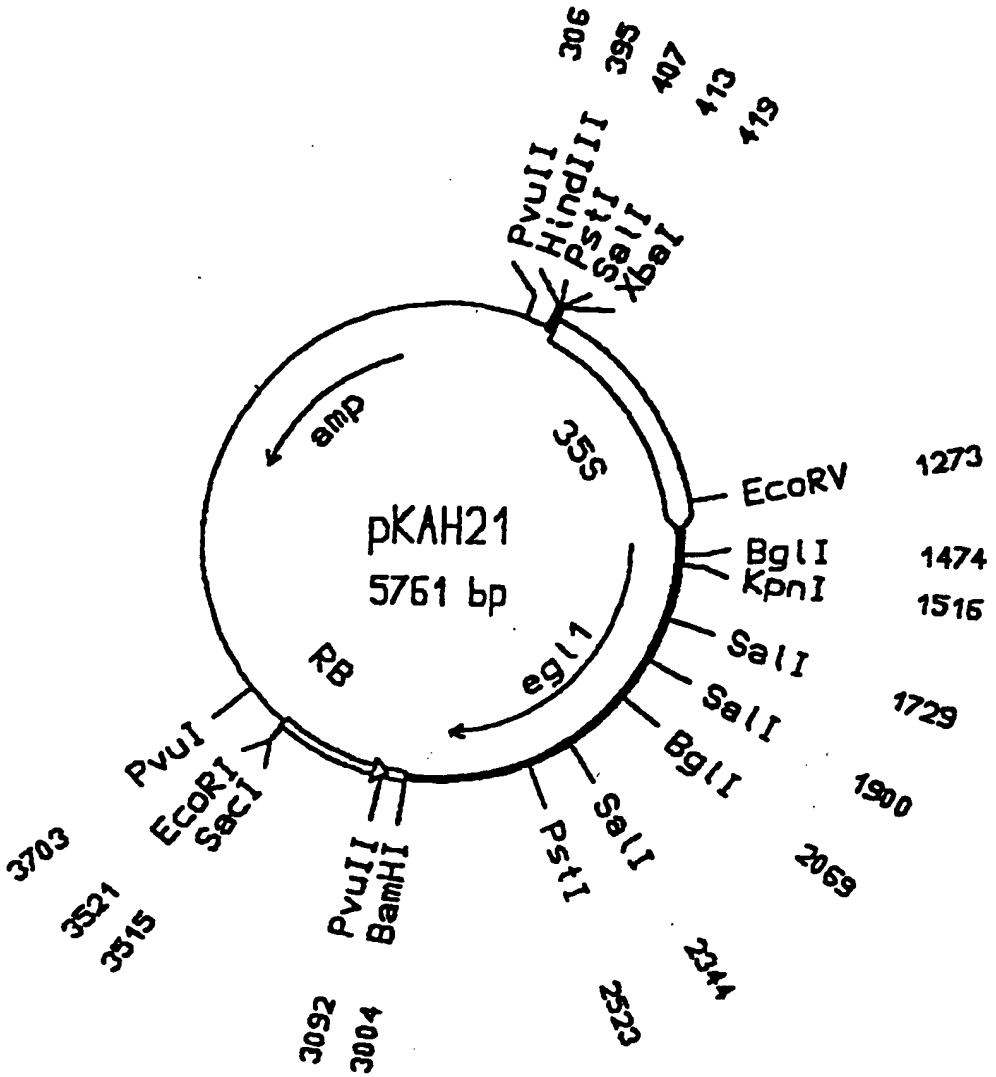
Figure 2A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XbaI
EcoRV
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BglI
KpnI
1474
1515
SalI
SalI
1729
BglI
1900
SalI
PstI
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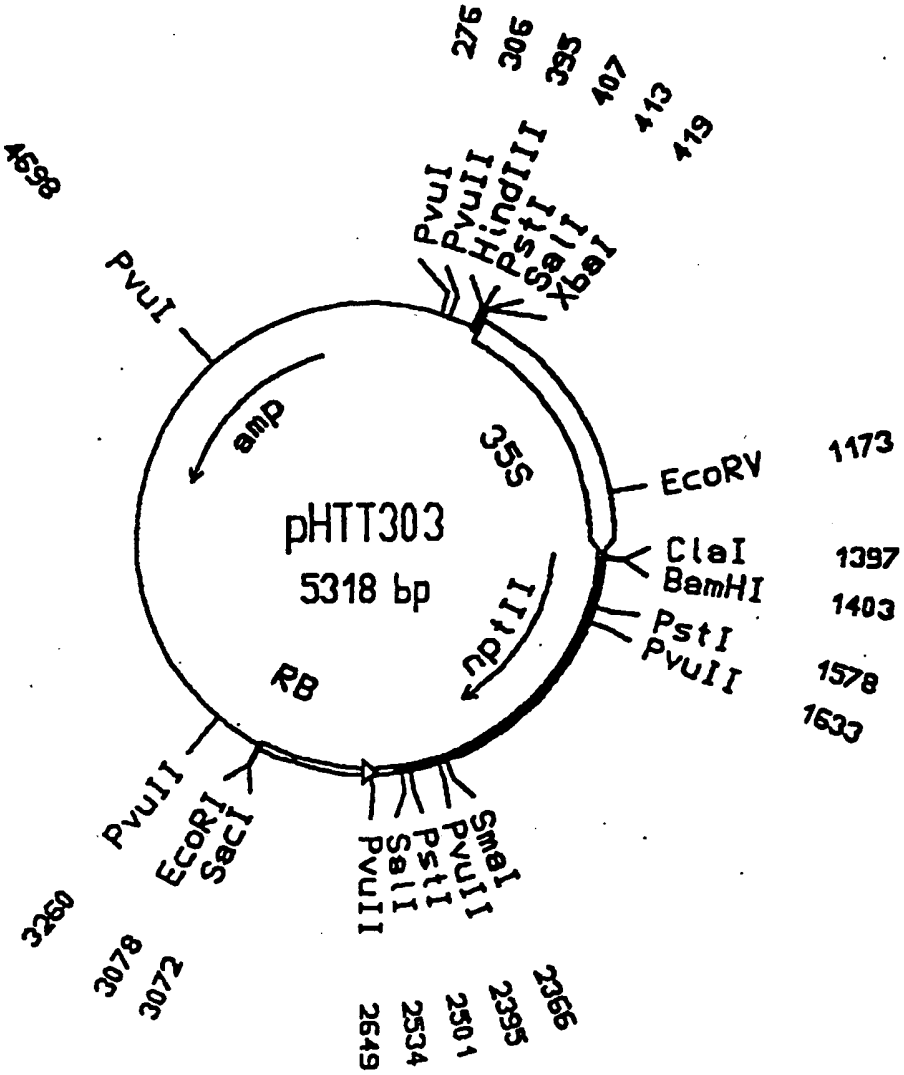
3515
3521
3703

Figure 2B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SalI
XbaI
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EcoRV
1397
ClaI
1403
BamHI
PstI
1578
PvuII
1633
SmaI
PvuII
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SalI
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SacI
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3078
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PvuI
4698

Figure 2C

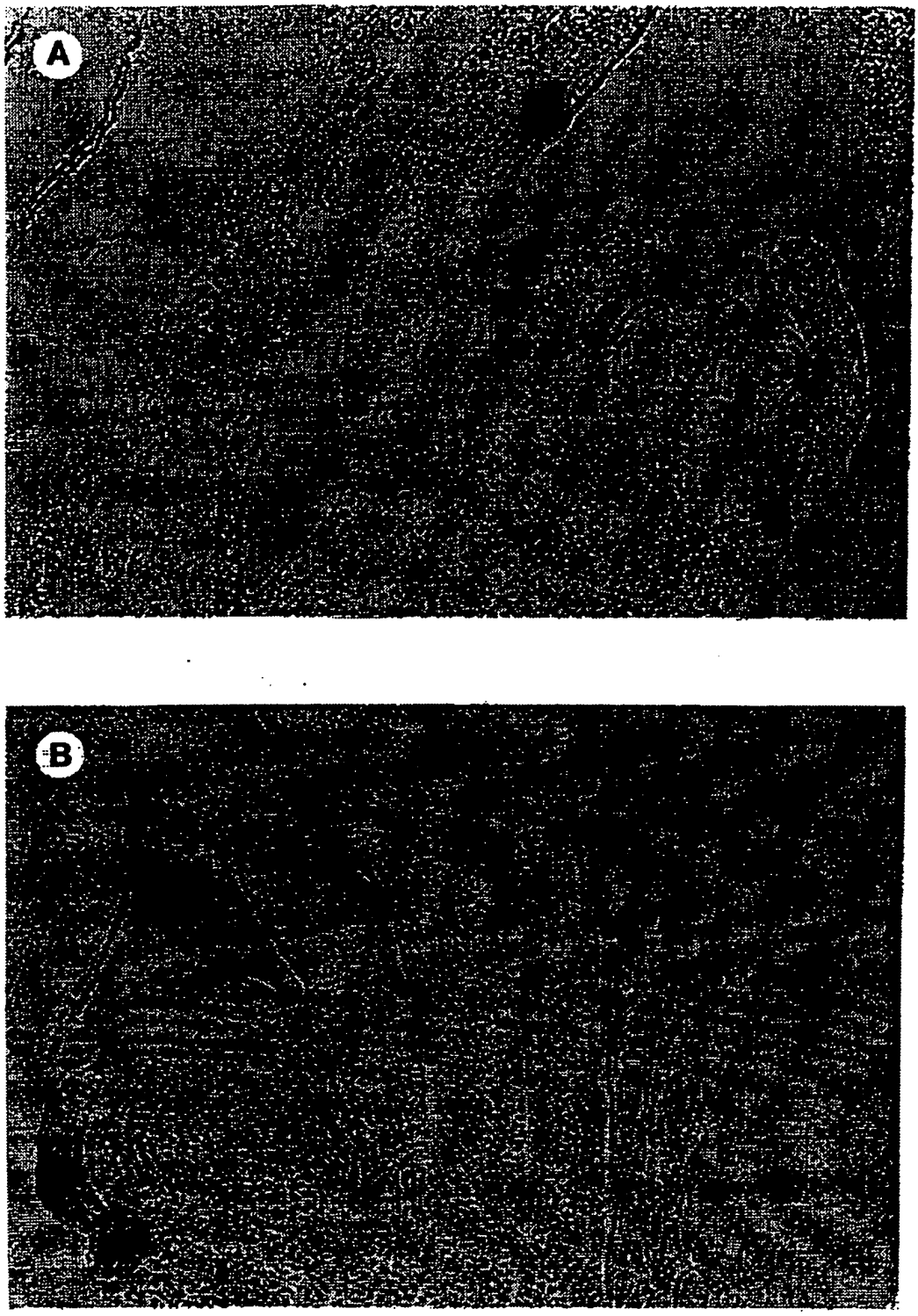


Figure 3

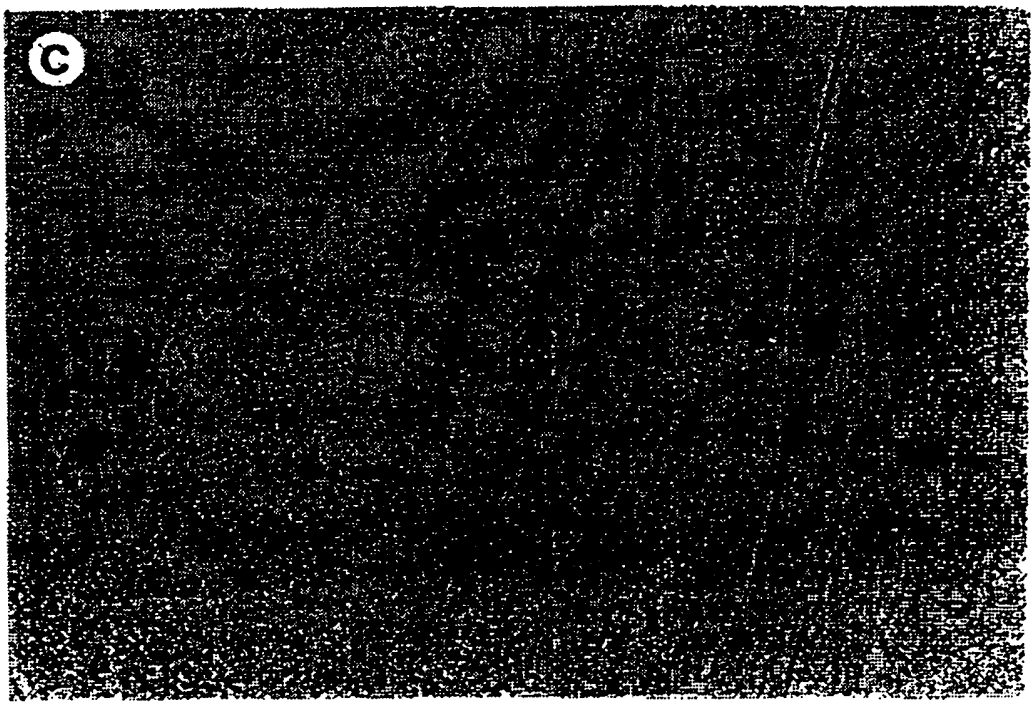
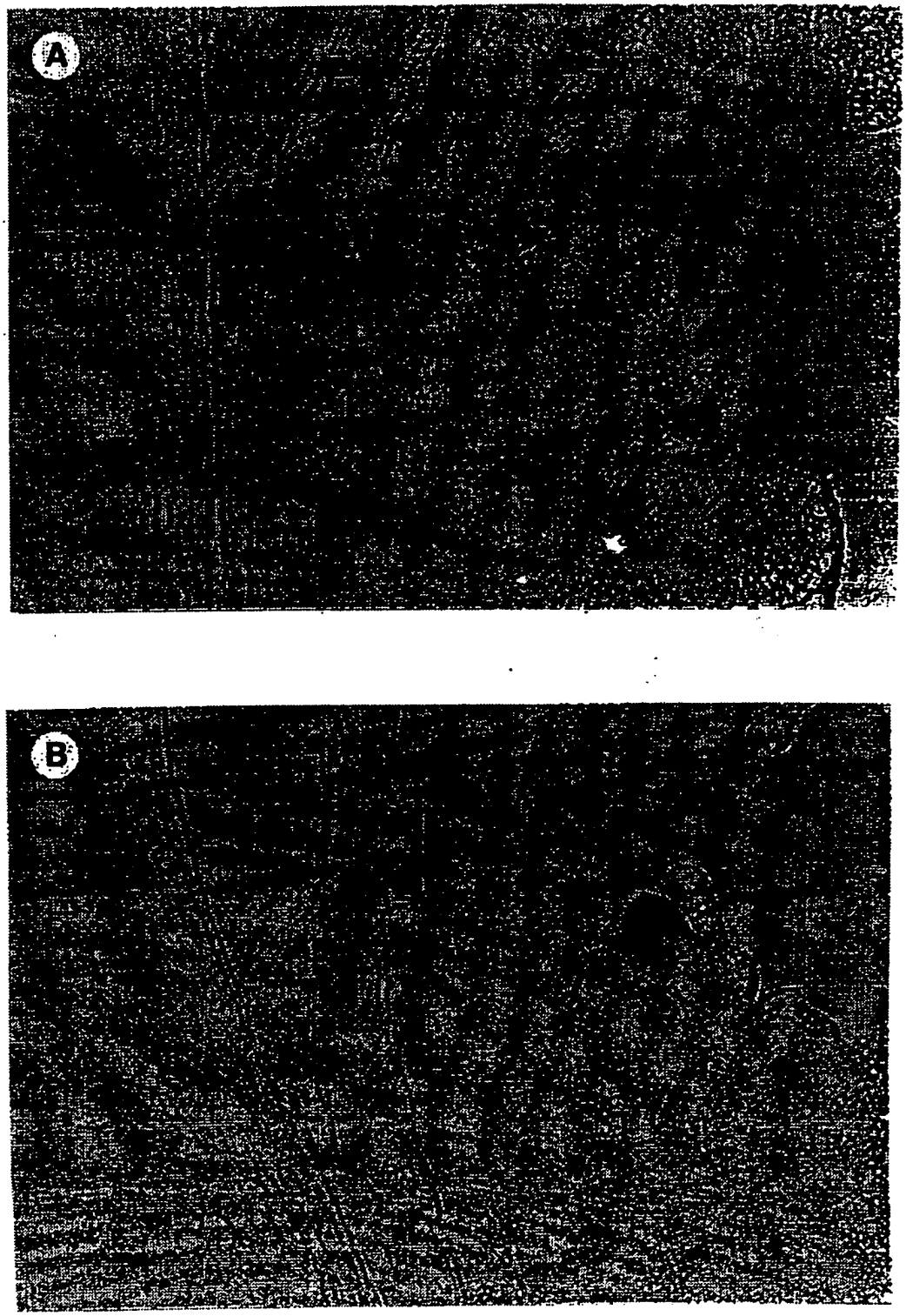


Figure 3



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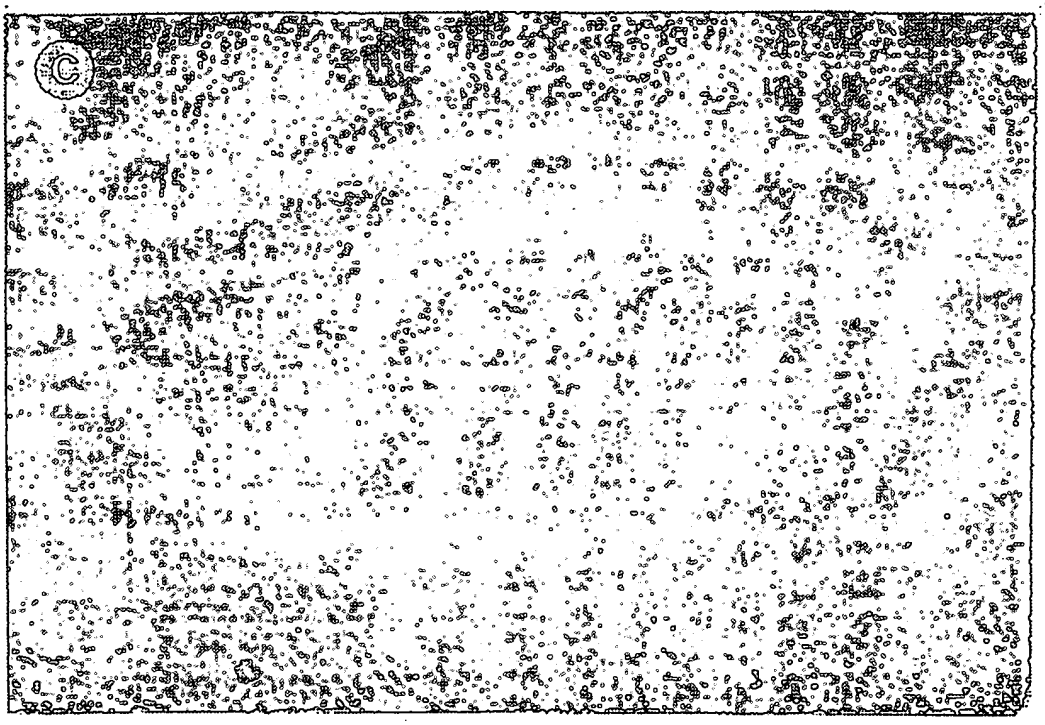

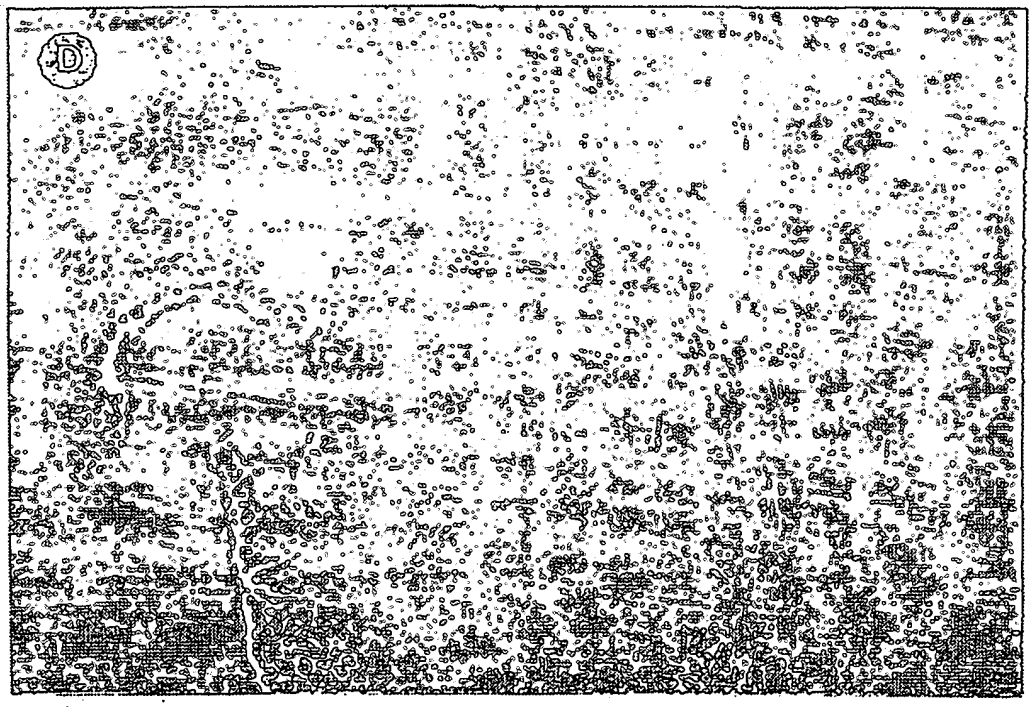

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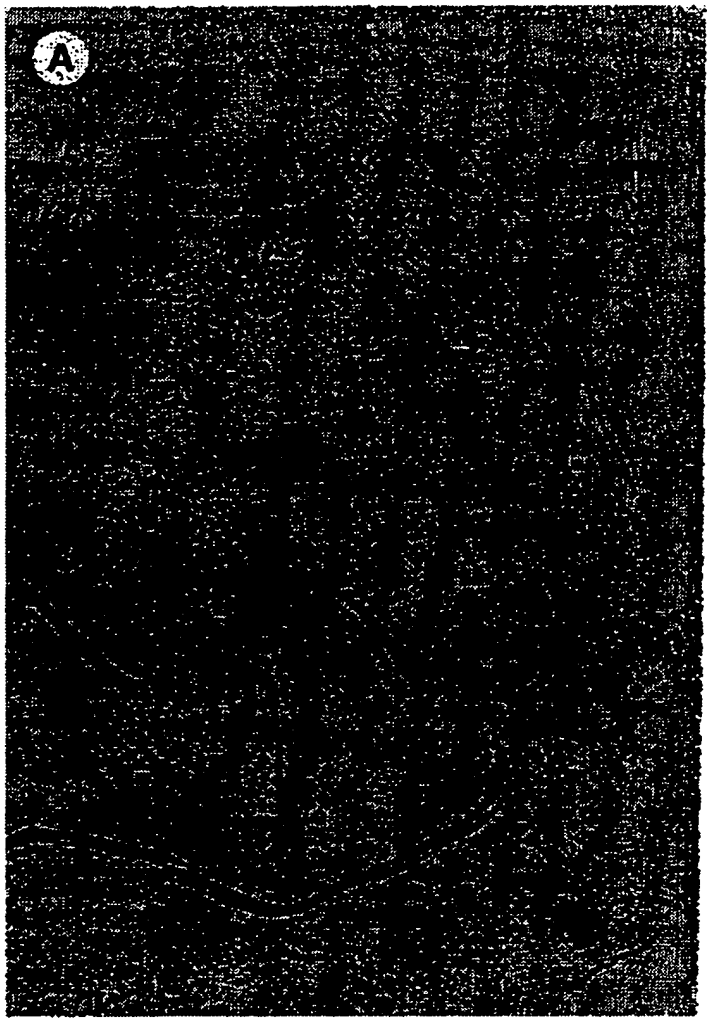
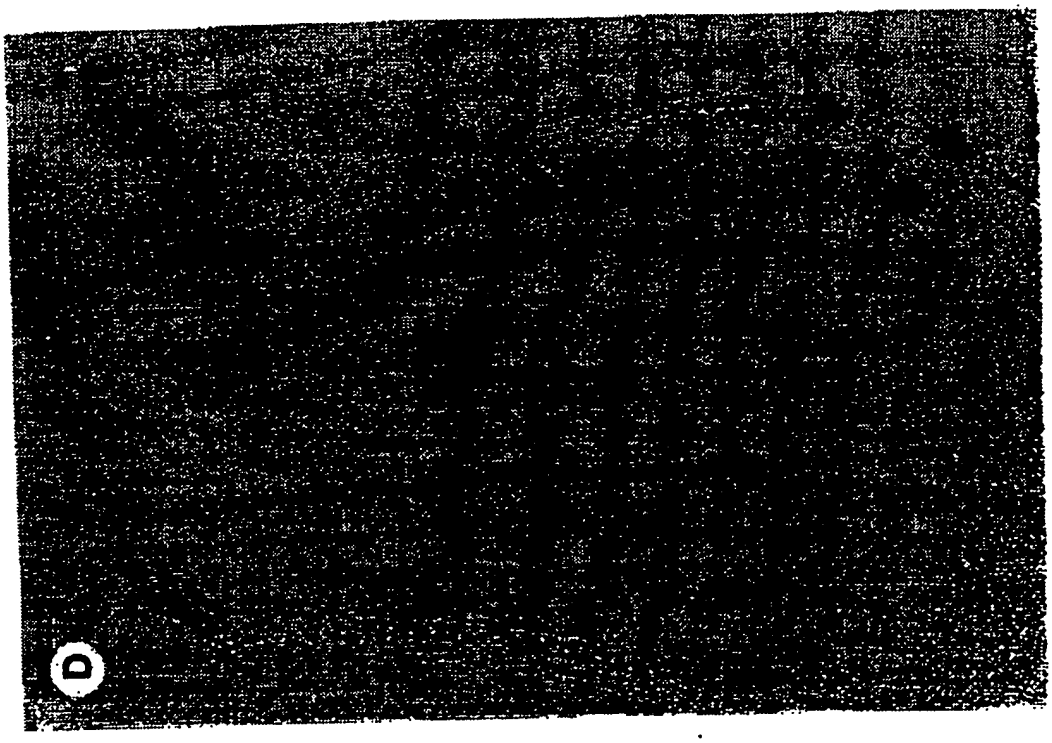
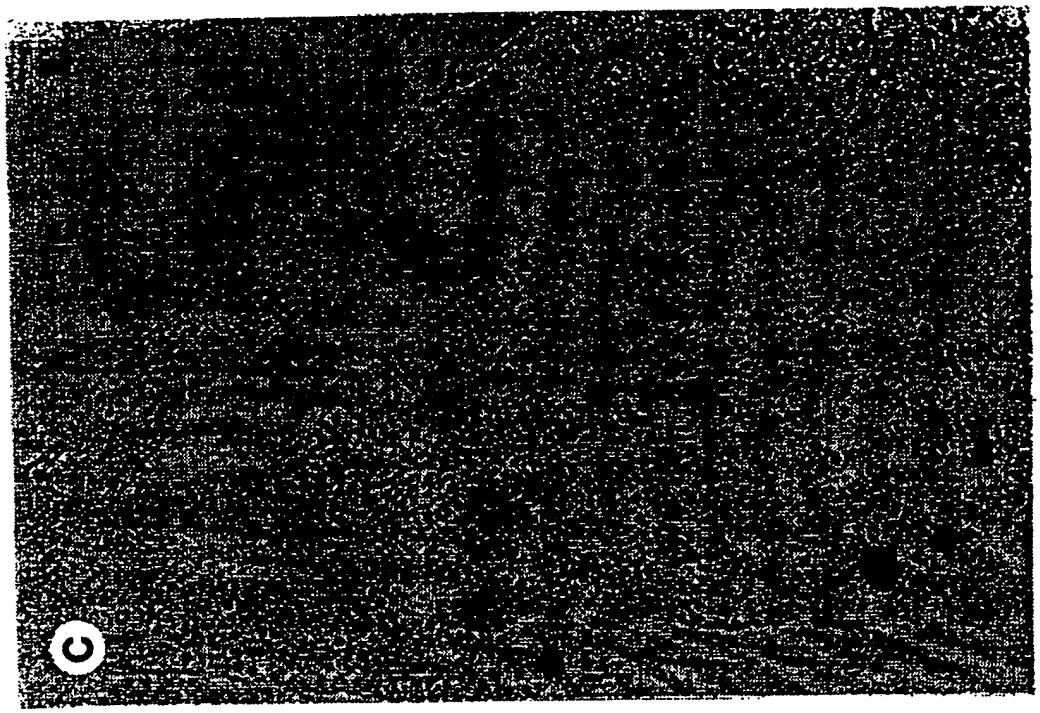


Figure 5

Figure 5





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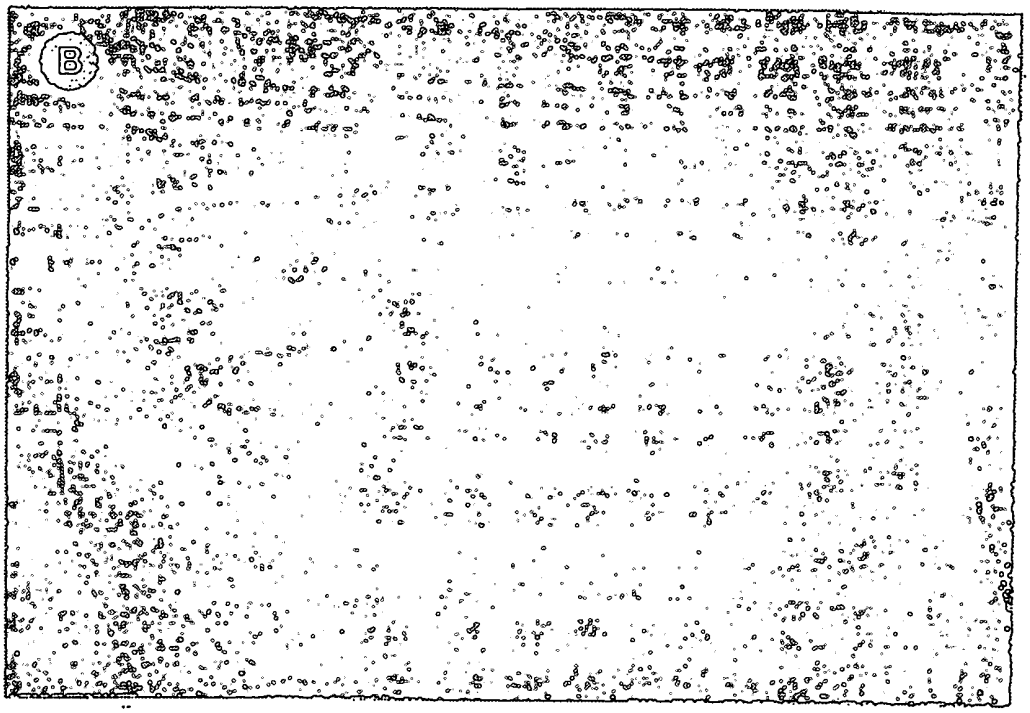

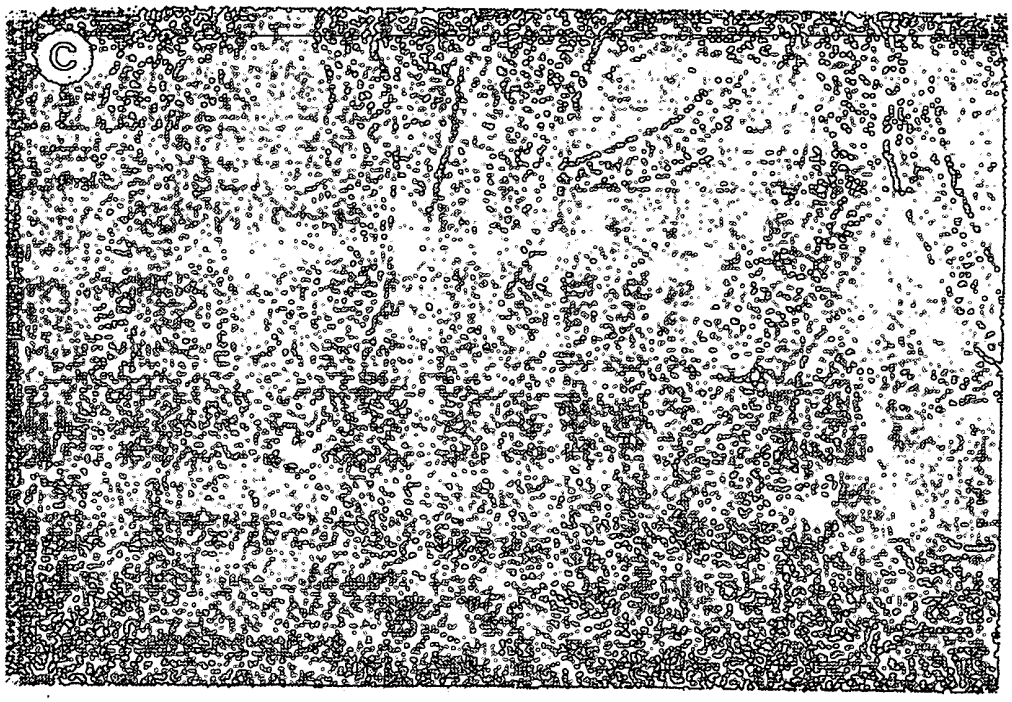

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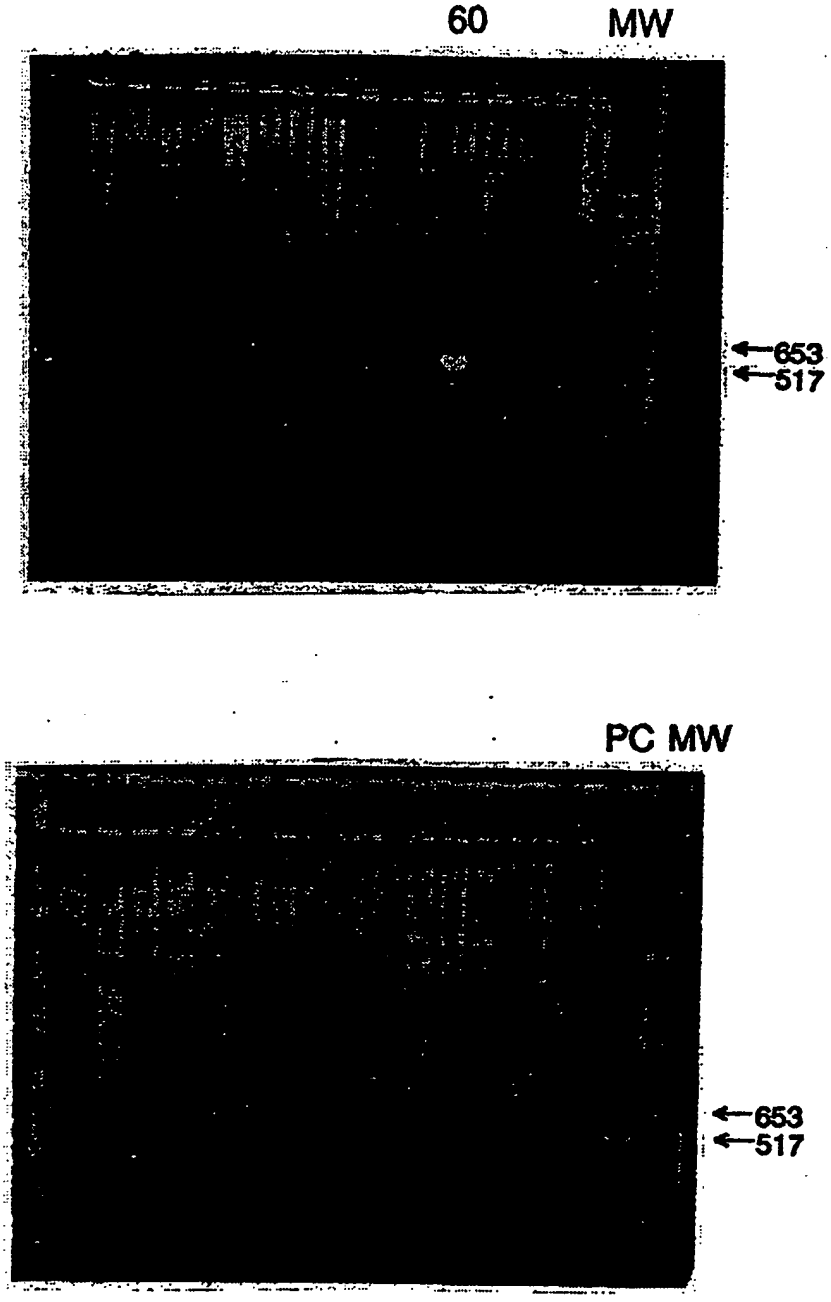
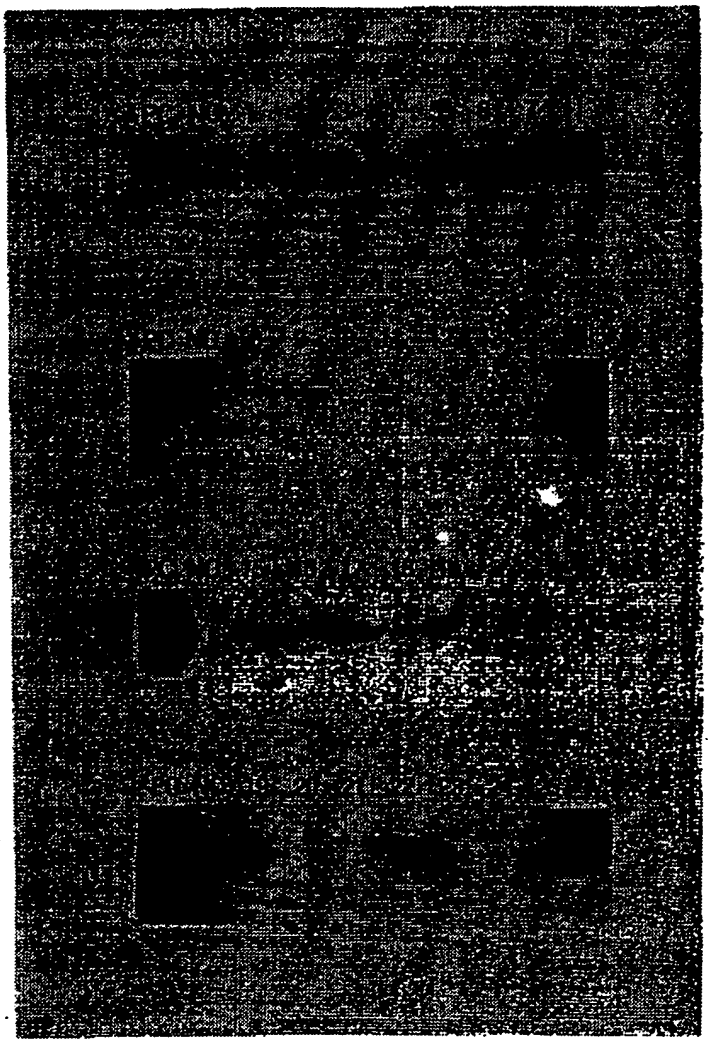


Figure 7

Lane 60 : positive sample

Lane PC : positive control

Lane MW : molecular weight markers



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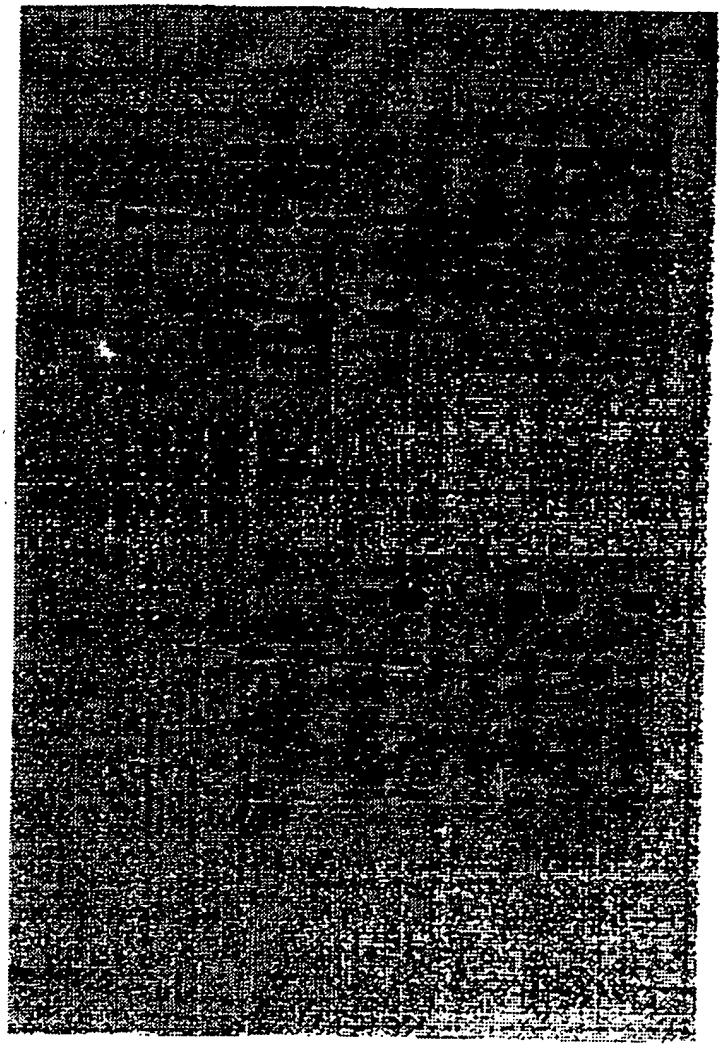

Figure 9

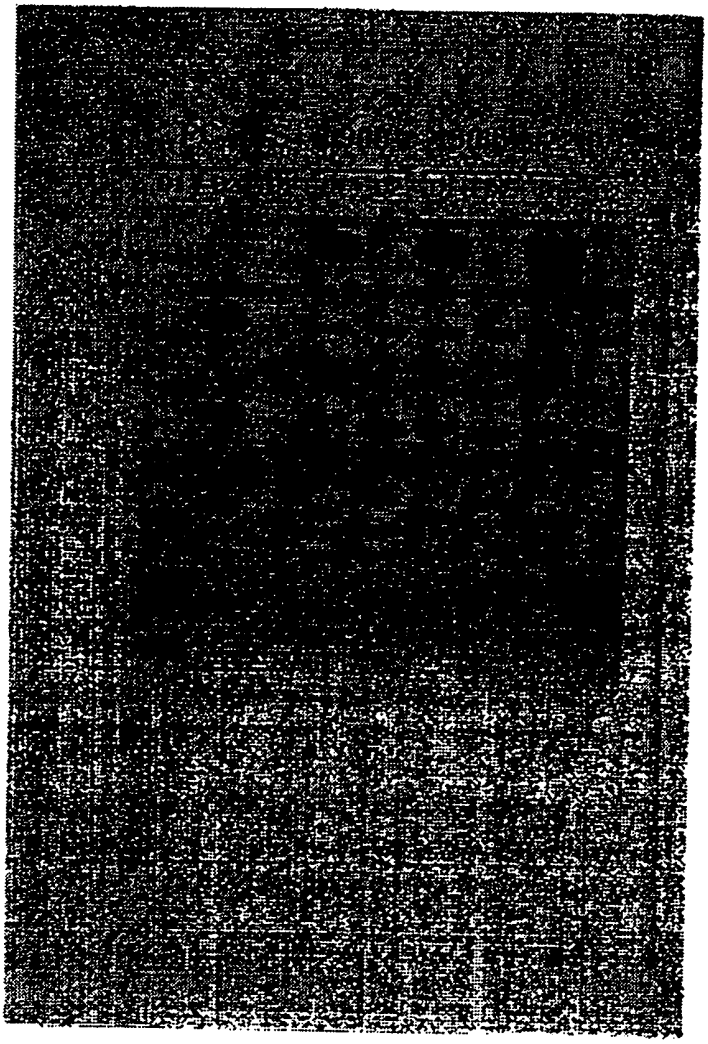

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 94/00365

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/82, C12N 15/83, A01H 4/00, A01H 5/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, BIOSIS, DBA, WPI, CLAIMS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9220809 (AGRACETUS, INC.), 26 November 1992 (26.11.92), see page 5, lines 27-34, page 6, line 25 - page 7, line 34, page 15, line 1 - page 20, line 26 and examples	1-7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1994	05 -12- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Jonny Brun Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

29/10/94

International application No.
PCT/FI 94/00365

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9220809	26/11/92	AU-A- 1905292	30/12/92
		EP-A,A- 0539563	05/05/93
		JP-T- 6500474	20/01/94